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Molecular analysis of the interaction between HPV type 16 E6 and human E6-associated protein

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Abstract The complex formed between the human papillomavirus type 16 E6 protein and human E6-associated protein, which combine to ubiquitylate and degrade p53, has been studied by chemical crosslinking. Analysis of the interactions of proteins purified from *Escherichia coli* as well as proteins expressed in insect cells indicates that, while E6 has the capacity to form dimers, E6 and E6-associated protein interact as two monomers to form a heterologous dimer.

Key words: Human papillomavirus; E6; E6-associated protein; Crosslinking

1. Introduction

Human papillomaviruses (HPV) have been implicated in the development of cervical carcinomas since the discovery of viral DNA in cervical cancer tissue and cell lines [1–3]. However, the transforming properties of the virus are restricted to a small region of the viral genome which encodes the E6 and E7 open reading frames (ORFs) [4,5]. E6 is a small, highly basic protein which binds to the p53 tumour suppressor protein [6] and induces its degradation via the ubiquitin dependent proteolysis pathway [7]. Inactivation of p53 is a common mechanism involved in the development of cancer and it has been demonstrated that, where HPV positive cancers retain wild-type p53 which is inactivated by E6, HPV negative carcinomas express a mutant p53 [8,9].

The degradation of p53 requires that E6 interacts with a cellular protein, E6-associated protein (E6-AP), because neither protein will bind to p53 without the other [10,11]. E6 and E6-AP, in concert, fulfil the role of a ubiquitin protein ligase in the ubiquitin degradation pathway [12]. This pathway (reviewed in [13,14]) involves the activation of ubiquitin by the formation of a high energy thioester bond with the ubiquitin activating enzyme E1. The activated ubiquitin is then transferred to a ubiquitin conjugating enzyme (E2) which was originally thought to catalyse the transfer of ubiquitin to the substrate protein, the recognition of which often required a third protein, a ubiquitin protein ligase (E3). However, recent evidence suggests that in some cases the E3 may be directly involved in the transfer of ubiquitin to the substrate [15,16]. This certainly seems to be the case in the E6 mediated proteolysis of p53 where ubiquitin is transferred from a specific E2 to E6-AP and ultimately to p53 [16,17].

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Most of the information regarding the E6/E6-AP ubiquitylation of p53 has been acquired from in vitro data and the interaction of E6 and E6-AP has yet to be confirmed in vivo. This is partly due to a lack of good antibodies to both E6 and E6-AP which, in turn, may be a consequence of using impure protein preparations and also the frequent use of fusion proteins that can mask potentially dominant epitopes. This latter point is an important factor in the development of antibodies against E6. We have cloned the cDNA of E6-AP from the cervical carcinoma cell line CaSki and have overexpressed both E6 and E6-AP in *Escherichia coli* in a soluble form. The two proteins have been purified to near homogeneity from soluble bacterial extracts and were used to produce highly specific, polyclonal antibodies in rabbits. Using the purified proteins and proteins expressed in insect cells, chemical crosslinking analyses have been carried out to examine the stoichiometry of the interactions of E6 and E6-AP in vitro and ex vivo.

2. Materials and methods

2.1. Plasmid constructions

The sequence for E6 was amplified from the reference viral clone by PCR (nts. 104–559) [18] and inserted into pT7Blue to form pT7E6. The cDNA for E6-AP was amplified, by RT-PCR of RNA isolated from CaSki cells, in two separate but overlapping halves (nts. 40–1593 and 1183–2598 from the sequence published by Huibregtse et al. [11]). The products of the RT-PCR were ligated into pT7Blue to produce pT7APA and pT7APB. The full length construct, pT7E6-AP, was constructed by ligating the *Bam*HI-*Bsm*I restriction fragment of pT7APB into pT7APA that had been cut with the same enzymes. From their respective pT7Blue clones the E6 and E6-AP ORFs were ligated into the bacterial expression vector pET15b to generate pETE6 and pETE6-AP. E6 and E6-AP were also sub-cloned into a previously modified baculovirus transfer vector pVL941 [19]. The human p53 cDNA was amplified by PCR from the clone pT7-7Hup53 [20] and ligated directly into pT7Blue to produce pT7p53. The entire nucleic acid sequences of all the clones generated were sequenced using an ABI 373A automated sequencer to verify that they were wild-type.

2.2. Protein expression and purification

E6 expression was induced in the host strain BL21(DE3)pLysS, grown in LB medium, by the addition of 1 mM IPTG for 5 h at 25°C. Protein was purified by using immobilised metal affinity (IMAC), cation exchange and size exclusion chromatography techniques. The histidine tag was also removed by thrombin cleavage. E6-AP expression was induced in BL21(DE3)pLysS, grown in LB medium, by the addition of 1 mM IPTG for 3 h at 25°C. The first purification step was by IMAC, followed by ammonium sulphate precipitation and then anion exchange and size exclusion chromatography.

2.3. Production of antisera and immunoblot analysis

Antisera specific for HPV 16 E6 and E6-AP were prepared by injecting New Zealand White rabbits subcutaneously with 0.5 mg of purified protein emulsified in an equal volume of Freund's adjuvant. Antisera were affinity purified using AffiGel 10 (BioRad) according to

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the manufacturer's instructions. E6-AP was coupled to the matrix in the presence of 80 mM $CaCl_2$. Immunoblot analysis was carried out essentially as described by Sanders et al. [19], and the affinity purified antisera were used at dilutions of 1:5000.

2.4. Expression of recombinant proteins in insect cells

Spodoptera frugiperda Sf21 cells were maintained in Grace's insect cell medium (Gibco BRL) supplemented with 10% fetal calf serum at 27°C. Cells were routinely adapted from monolayer to spinner culture and were used for the production and propagation of the recombinant baculoviruses. BTI-TN-5B1-4 (Hi5) cells were grown in monolayer under the same conditions and were used for the expression of proteins only. Recombinant baculoviruses were generated, purified and propagated according to Sanders et al. [19], and designated rvE6 and rvE6-AP. Hi5 cells were infected at a multiplicity of infection of 5 or greater and maintained at 27°C for the required length of time.

2.5. p53 degradation assays

p53 RNA was transcribed in vitro from linearised pT7p53 and translations were performed in rabbit reticulocyte lysate (RLL) or wheat germ extract (WGE) (Promega) in the presence of [^{35}S]methionine/cysteine (ProMix, Amersham International, plc) according to the manufacturers' instructions. To prevent subsequent translation, in vitro translation lysates were incubated with RNase added to 1 μg/ml at room temperature for 5 min. 2–10 μl of p53 translated in RLL was mixed with 1 μl of various dilutions of pure E6 (1 mg/ml) and incubated at 30°C for 1 h. WGE degradation assays were carried out as described by Huibregtse et al. [11], but with in vitro translated HPV 16 E6 and E6-AP replaced by an equivalent amount of WGE and the purified proteins. Samples were then mixed with 200 μl of buffer A (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1% (v/v) Nonidet P-40) before immunoprecipitating with anti-p53 antibody pAb421 (Oncogene Science). Reaction products were analysed by SDS-PAGE and autoradiography.

2.6. Analysis of protein-protein interactions by chemical crosslinking

Purified HPV 16 E6, at a concentration of 1 mg/ml, was diluted 1/5 in 25 mM HEPES, pH 7.9, 1 mM dithiothreitol (DTT), to reduce the concentration of NaCl. Proteins, at 200 μg/ml, were incubated on ice for 30 min in the presence of 1 mM disuccinimidyl suberate (DSS, resuspended in DMSO). Reaction volumes were equated, where necessary, with 25 mM HEPES, pH 7.9, 100 mM NaCl, 1 mM DTT. Reactions were quenched by the addition of Tris-HCl, pH 7.5 to a final concentration of 50 mM and a further 15 min incubation on ice. To carry out crosslinking analysis on insect cell lysates, Hi5 insect cells at 48 h post infection were harvested, washed in PBS and lysed in lysis buffer (25 mM HEPES, pH 7.9, 100 mM NaCl, 0.5% Nonidet P-40, 1 mM DTT, 1 mM phenylmethylsulphonyl fluoride, 1 μM pepstatin, 10 μg/ml E-64) for 20 min before clarifying by centrifugation at 50 000×*g* for 20 min at 4°C. Protein concentrations were equated by the addition of lysis buffer. Reactions were carried out as above but using bis(sulfosuccinimidyl) suberate (BS^3, resuspended in dH_2O) instead of DSS and volumes were equated, where necessary, with lysates from wild-type infected insect cells. Protein samples were analysed by SDS-PAGE followed by immunoblotting.

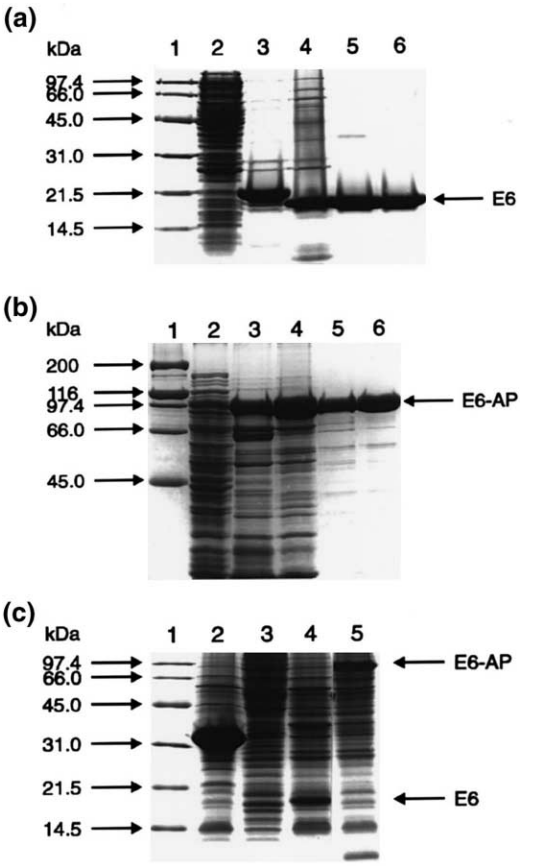

Fig. 1. a: Expression and purification of HPV 16 E6 expressed in *E. coli*, as described in Section 2, analysed on a 15% SDS-polyacrylamide gel. Lane 1, molecular weight marker proteins; lane 2, total soluble protein from *E. coli* transformed with pETE6; soluble protein following: lane 3, immobilised metal affinity chromatography; lane 4, thrombin cleavage; lane 5, cation exchange chromatography and lane 6, soluble, pure E6 following gel filtration chromatography. b: Expression and purification of E6-AP expressed in *E. coli* analysed on a 10% SDS-polyacrylamide gel. Lane 1, molecular weight marker proteins; lane 2, total soluble protein from *E. coli* transformed with pETE6-AP; soluble protein following: lane 3, immobilised metal affinity chromatography; lane 4, ammonium sulphate precipitation; lane 5, anion exchange chromatography and lane 6, soluble, pure E6-AP following gel filtration chromatography. c: Expression of E6 and E6-AP in Hi5 insect cells. Insect cells were infected with recombinant baculoviruses, harvested at 72 h post infection and total cell protein was analysed by SDS-PAGE on a 15% gel. Lane 1, molecular weight marker proteins; lane 2, wild type AcMNPV infected cells; lane 3, mock infected cells; lane 4, rvE6 infected cells; lane 5, rvE6-AP infected cells.

3. Results

E6 and E6-AP were cloned into the bacterial expression vector pET15b via the cloning vector pT7Blue and all clones were completely sequenced. They were then expressed in *E. coli* and soluble protein was purified to near homogeneity as described in Section 2 (Fig. 1). Recombinant baculoviruses expressing E6 and E6-AP were also generated using their respective pVL941 clones. When Hi5 insect cells were infected with one of the baculoviruses and expression levels analysed by SDS-PAGE and Coomassie staining, proteins migrating at the predicted molecular weights for E6 and E6-AP were clearly visible (Fig. 1).

To confirm that the proteins had retained a native, biochemically active conformation, their ability to facilitate p53 proteolysis was determined. When p53 was translated in reticulocyte lysate, which possesses intrinsic E6-AP activity [10], its degradation was induced by the addition of either purified E6 or E6-containing insect cell lysates (Fig. 2a). When translated in WGE, which has no E6-AP activity [10], p53 degradation was promoted, as predicted, upon addition of both E6 and E6-AP (Fig. 2b). Data are shown for the purified proteins but identical results were obtained using insect cell lysates.

Using the purified, biologically active proteins we set out to investigate the interactions of E6 and E6-AP in vitro by employing chemical crosslinking techniques which would permit determination of the molecular weights of complexes formed by comparison with molecular weight standards. To enable us to manipulate small amounts of protein and to determine the

precise components of the complexes formed, the gels were subjected to Western analysis.

When E6 alone was treated with the crosslinker DSS and analysed by immunoblotting there was evidence for specific dimerisation (Fig. 3a, lane 2). On the addition of E6-AP, a high molecular weight band was detected which migrated slightly faster than the 140 kDa molecular weight marker (Fig. 3a, lane 4). This complex therefore represents the E6:E6-AP complex. When a similar analysis was performed with E6-AP, followed by immunoblotting with the E6-AP antibody, a 100 kDa species corresponding to E6-AP alone was identified (Fig. 3b, lane 2). Upon the addition of E6, an additional, slower moving band, migrating at 120 kDa, was detected which represents the E6:E6-AP complex (Fig. 3b, lane 4). These data suggest that E6 and E6-AP interact as two monomers despite the ability of purified E6 to form a homodimer.

To establish that the complex identified on combining purified E6 and E6-AP was specific and not an artifact we sought to detect a similar high molecular weight complex in a protein lysate where other protein-protein interactions could take precedence. Therefore, a similar analysis was carried out using lysates from insect cells infected with recombinant baculoviruses expressing biologically active E6 and E6-AP. Clearer results were obtained in this system using the water soluble analogue of DSS, BS^3 (data not shown).

There was again evidence for formation of an E6 dimer when a lysate containing E6 only was treated with crosslinker (Fig. 4a, lane 2). However, a higher molecular weight band was visible (complex A), running at approximately 100 kDa. On combining the lysates from cells infected with rvE6 or rvE6-AP a second higher molecular weight band (complex B) was detected which migrated more slowly than that present with E6 alone (Fig. 4a, lane 6). Complex B ran at the same molecular weight by SDS-PAGE as the high molecular weight complex observed with the purified proteins. Interestingly, complex A was not observed when the same analysis was carried out on a lysate that had been coinfected with rvE6 and rvE6-AP (Fig. 4a, lane 4). If the lysates from the cells infected with rvE6 or rvE6-AP were mixed and preincubated,

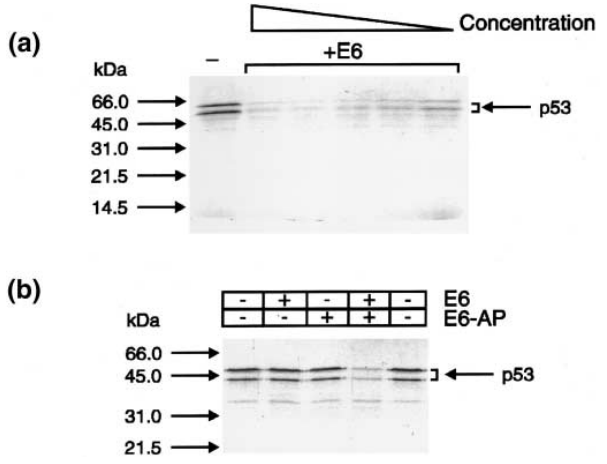

Fig. 2. Degradation of in vitro translated p53 induced by E6 and E6-AP purified from *E. coli*. a: p53 was translated in reticulocyte lysate and pure E6 was added at dilutions down to 1:500. b: p53 was translated in WGE and pure E6 and E6-AP were added, as indicated. All reactions were carried out as described in Section 2 and analysed by SDS-PAGE on a 12% gel and autoradiography.

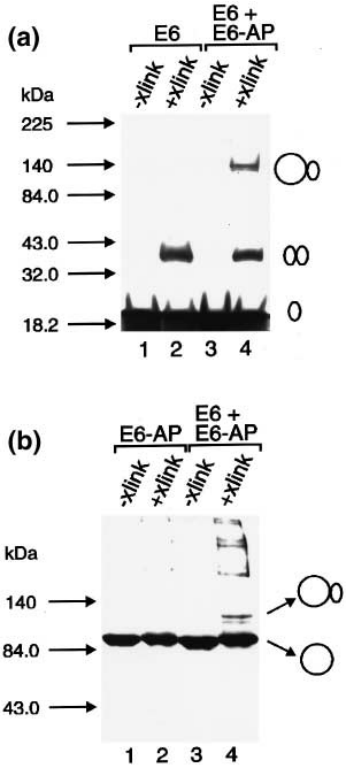

Fig. 3. Crosslinking E6 and E6-AP proteins purified from *E. coli*. a: E6 (lanes 1 and 2) or E6 with E6-AP (lanes 3 and 4) were incubated in the presence of DSS (lanes 2 and 4) or DMSO alone, as a control, (lanes 1 and 3). Samples were analysed on a 5–20% gradient gel followed by Western analysis with the E6 specific antiserum. b: E6-AP (lanes 1 and 2) or E6 with E6-AP (lanes 3 and 4) were incubated in the presence of DSS (lanes 2 and 4) or DMSO alone (lanes 1 and 3). Samples were analysed on a 6% gel followed by Western analysis with the E6-AP specific antiserum. Positions of molecular weight reference markers are indicated. Circles represent E6-AP and small ovals represent E6.

prior to crosslinking, the relative yield of complexes A and B was not altered (data not shown).

When a similar analysis was carried out on lysates expressing E6-AP followed by Western analysis with an E6-AP antibody (Fig. 4b, lane 2) a major band at 100 kDa was identified, as expected. On mixing lysates from rvE6 and rvE6-AP infected cells (Fig. 4b, lanes 5 and 6) or when analysing lysates from cells coinfected with both rvE6 and rvE6-AP (Fig. 4b, lanes 3 and 4) a novel species, also migrating at the position of an E6:E6-AP heterodimer, was detected.

4. Discussion

There is much in vitro evidence demonstrating that, during the degradation of p53 by the ubiquitin dependent proteolysis pathway, E6 and E6-AP function as a ubiquitin protein ligase in the ubiquitylation cascade. However, an interaction between the two proteins has not been demonstrated in vivo and the results of previous studies caution a direct correlation between in vitro and in vivo data [21]. There are also no direct data regarding the stoichiometry of any of these interactions. As a result we set out to examine the interactions of HPV 16 E6 and E6-AP in vitro and ex vivo, i.e. overexpressed in insect cells.

The cDNA of E6-AP was cloned from the cervical carcino-

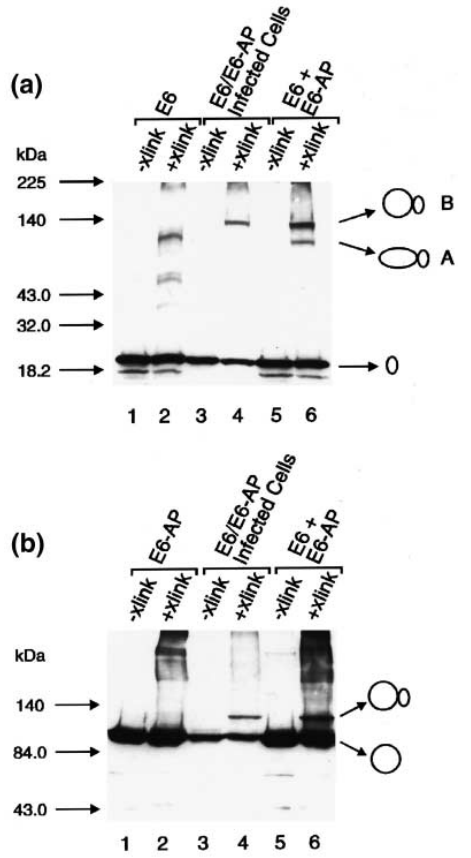

Fig. 4. Crosslinking insect cell lysates. a: Lysates from rvE6 infected cells (lanes 1 and 2), lysates from cells coinfected with rvE6 and rvE6-AP (lanes 3 and 4) or lysates from rvE6 infected cells mixed with lysates from rvE6-AP infected cells (lanes 5 and 6) were incubated in the presence of BS^3 (lanes 2, 4 and 6) or dH_2O (lanes 1, 3 and 5). Samples were analysed on a 5–20% gradient gel and analysed by immunoblotting with the E6 antiserum. b: Lysates from rvE6-AP infected cells (lanes 1 and 2), lysates from cells coinfected with rvE6-AP and rvE6 (lanes 3 and 4) or lysates from rvE6-AP infected cells mixed with lysates from rvE6 infected cells (lanes 5 and 6) were incubated in the presence of BS^3 (lanes 2, 4 and 6) or dH_2O (lanes 1, 3 and 5). Samples were analysed on a 6% gel and analysed by immunoblotting with the E6-AP antiserum. Positions of molecular weight reference markers are indicated. Circles represent E6-AP, small vertical ovals represent E6 and flat ovals represent a cellular or viral protein.

ma cell line CaSki on the basis that continuous E6 function, including the degradation of p53, is required for the maintenance of the neoplastic phenotype in these cells [22,23]. This evidence implies that these cells will have a functional E6-AP. There are no data regarding expression of the original full length cDNA but we were able to overexpress soluble, full length protein in *E. coli* and purify it to virtual homogeneity.

HPV 16 E6 was also overexpressed in *E. coli* and soluble protein was purified to near homogeneity. Cleavage of the short His tag from the protein resulted in a non-fusion product, except for 3 amino acids. This is important because most of the studies on E6 have involved the use of large, mainly N-terminal, fusions such as glutathione *S*-transferase or maltose binding protein. It is very likely that these fusion proteins will have an effect on the ability of the small, 18 kDa E6 to interact with other proteins. These effects could be allosteric or simply obstructive. Other workers have also found that E6 is highly insoluble and as a result have applied denaturation/renaturation techniques to obtain purified protein. It is well known that these harsh treatments can, at the very least, partially inactivate proteins.

The presence of a fusion protein could also have a crucial effect on the production of antibodies against E6. This could be particularly important in the case of an N-terminal fusion because the N-terminus of E6 has been demonstrated to be highly antigenic [24]. Anti-E6 antibodies are notoriously poor and other workers have found that even monoclonal antibodies raised against E6 crossreacted with normal human tissue [25]. Although there was still an element of crossreactivity with the E6 antiserum described here, it did not crossreact with HPV 18 E6 (data not shown).

To analyse the interactions of E6 and E6-AP by chemical crosslinking, crosslinkers were chosen on the basis of their spacer arm length (11.4 Å), a length that should avoid crosslinking non-specific interactions without prohibiting those that are specific. DSS has also been successfully applied to identifying other protein-protein interactions [26] and BS^3 is its water soluble analogue. Crosslinking analysis of the purified proteins provides strong evidence for the existence of E6 dimers, a possibility that has only previously been suggested based on indirect evidence [27,28]. E6 does not resolve as a dimeric species by size exclusion chromatography (data not shown) which may be a consequence of the high salt running conditions used. This suggests that the E6 dimers are not very stable, especially when compared to the stability of dimers of other papillomavirus proteins, such as those formed by BPV E2 [29].

When pure E6 and E6-AP were crosslinked, a high molecular weight species was observed which corresponds to the E6:E6-AP complex. Comparison with molecular weight standards indicates that this complex almost certainly represents one molecule of E6 interacting with one molecule of E6-AP and is in agreement with the suggestion by Huibregtse et al. [11] that the two proteins interact with a ratio of 1:1. Although crosslinking can effect the mobility of proteins in SDS polyacrylamide gels by producing protein masses with unusual shapes, the small size of E6 relative to E6-AP significantly reduces this possibility. The doublet at the position of the E6:E6-AP complex in Fig. 3b probably represents two isoforms of the complex as a result of differential crosslinking.

The crosslinking experiments with insect cell lysates, where non-specific contacts between E6 and E6-AP should be eliminated and any post-translational modifications of the proteins will occur, was used to confirm the specificity of the results seen with the purified proteins and to show that the same interactions take place in vivo. Crosslinking of lysates from cells expressing only E6 provided further evidence for the formation of an E6 dimer and confirms the specificity of the interaction detected with the pure protein (Fig. 4a). However, it indicated the preference for other protein:protein interactions in vivo as complex A presumably represents a complex of E6 with another cellular or viral protein. Although complex A was still present when lysates from cells expressing E6 or E6-AP were mixed together and analysed, there was also evidence for the E6:E6-AP complex (complex B). This high molecular weight species migrated to the same position as that seen with the crosslinked purified proteins. The ratio of these two complexes did not change with time of preincubation suggesting that both complexes were stable. Interestingly, when a lysate from cells coinfected with both rvE6 and

rvE6-AP was subjected to the same analysis only the E6:E6-AP complex (complex B) was evident and complex A was not observed. This suggests that when E6 is cotranslated with E6-AP, it preferentially forms a complex with E6-AP rather than with the cellular/viral protein. Alternatively, this observation may be a consequence of intracellular localisation of the proteins. If the cellular/viral protein is localised to a different compartment of the cell then it is possible that it only interacts with E6 once the cells are lysed. In contrast, assuming that E6 and E6-AP are colocalised in the cell, their complex may have preformed before the cell lysis and remained stable throughout the experiment. These possibilities can be investigated by carrying out a crosslinking analysis on the intact cells. Results obtained by crosslinking lysates and then analysing for the presence of E6-AP further confirmed the existence of the E6:E6-AP heterodimer (Fig. 4b).

The literature provides several examples of studies which have attempted to identify other proteins with which E6 interacts and/or alternative purposes for the protein. However, the only well understood function of E6, to date, is its capacity to accelerate the degradation of p53. The ability of single molecules of E6 and E6-AP to interact to form a heterodimer, both in vivo and in vitro, alongside the potential for E6 to form dimers, provides support for the possibility that E6 plays a further role in the life cycle of HPV, other than the degradation of p53, and that these other functions may depend upon supramolecular structural differences of the protein. The proteins and antibodies described here will be useful in further structural, functional and biochemical analyses of E6 and E6-AP and also in the study of their interactions with p53. Furthermore, chemical crosslinking may provide an alternative method for identifying the other proteins which interact with E6.

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